BIOPHARMACEUTICAL ASPECTS OF CORTICOSTEROID THERAPY IN PRETERM INFANTS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2003

This thesis is dedicated to my family for their constant love, encouragement and support of my goals.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Dr. Guenther Hochhaus, my mentor and advisor whose constant encouragement, endless patience and unfaltering support was instrumental in the successful completion of this dissertation. I would also like to thank the members of my supervisory committee (Drs. Hartmut Derendorf, Jeffrey Hughes and S. Khan) who individually, and as a group, provided excellent input and guidance during the course of my doctoral research.

I would also like to sincerely thank my friends in the lab (Boglarka, Intira, Kai, Manish, Sriks, Yaning) for their willingness to share their academic and personal experiences. I would also thank the secretaries of the department for their technical support.

I would like to thank my brother, Dr. Vivek Arya for his constant support and for always believing in me. I would not have even able to begin, much less complete, this great academic endeavor without his help.

I also wish to acknowledge my American "family" for making me feel at home away from home, providing insight into American culture, and sharing all the fun times.

Finally, I wish to extend my deepest gratitude to my parents for their constant love and encouragement. There is nothing without their presence.

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of the University of Florida in Partial Fulfillment of the
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December 2003

Chair: Guenther Hochhaus
Major Department: Pharmaceutics

Premeture birth is a major cause of infant mortality in the United States. The immaturity of the vital organs such as lungs necessitates the use of artificial respiratory support. The ensuing pulmonary damage predisposes the preterm infant to a wide array of medical complications such as chronic lung disease (CLD). The benefits of using systemic corticosteroids for the treatment/prevention of CLD in preterm infants are well documented. However, the concomitant observance of neurotoxic adverse effects in premature infants (and absent in adults), after systemic corticosteroid administration, have led to exploration of alternate routes of corticosteroid delivery. The administration of corticosteroids through the inhalation route has met with limited success, partly due to the rapid absorption of the corticosteroid from the lungs into the systemic circulation leading to loss of pulmonary targeting. Computer simulations have reiterated the importance of optimizing the drug release rate for improving pulmonary targeting. The overall objective was to study the biopharmaceutical factors such as brain permeability and pulmonary residence time that modulate the disposition of inhaled corticosteroids in preterm infants.

The role of p-glycoprotein transporters in modulating the brain permeability of inhaled corticosteroids was evaluated by assessing the brain and liver receptor occupancy in wild type and mdr1a mice after intravenous administration of TAP.

Ex vivo receptor binding assay was used for assessing pulmonary and systemic corticosteroid exposure in neonatal rats after intratracheal administration of triamcinolone acetonide phosphate (TAP) solution. To gain more insight into pulmonary residence time and pulmonary targeting, the neonatal rat model was used to determine the pulmonary targeting of poly (l-lactic acid) (PLA) coated budesonide.

Mdr1a mice showed significantly higher brain receptor occupancy than wild type mice, which suggests the pivotal role played by p-gp in modulating the brain permeability of corticosteroids. We did not observe pulmonary targeting after intratracheal administration of TAP. However, we observed significant brain receptor occupancy in neonatal rats that was in sharp contrast to minimal brain receptor occupancy in adult rats. Polymeric coated budesonide significantly higher pulmonary targeting as compared to uncoated budesonide.

Overall, the results underscore the urgent need to develop pulmonary targeted sustained-release delivery systems for corticosteroids in preterm infants. This will potentially result in an improved benefit-to-risk ratio of inhaled corticosteroid therapy for CLD.

CHAPTER1
BACKGROUND

Introduction

Preterm birth, observed in 7-10 % of all pregnancies in the United States, continues to be a major cause of infant morbidity and mortality (1). Medical complications arising due to prematurity result in significant health care costs (estimated to be $10 billion in the US annually), frequent hospitalizations and great emotional burden for the family.

The normal gestational age (number of completed weeks of pregnancy from the last menstrual period) of a full term baby is 40 weeks. Preterm (or premature) babies are born before 37 weeks of completed gestation. Although some preterm births are elective, a variety of factors such as previous preterm birth, uterine or cervical abnormalities, use of illicit drugs and low socio-economic status increase the risk of women delivering preterm.

Because of immature birth, the vital organs of the preterm infant such as the lungs and brain are not fully developed and are incapable of performing the vital functions required for healthy survival. Bolt et al. (2) reviewed lung development in premature infants. The human lung development can be classified into five distinct phases embryonic, pseudoglandular, canilicular, and saccular: and the alveolar phase (that continues after birth). These phases encompass the various stages of the pulmonary development process and are operative at distinct phases of gestation (e.g., the embryonic phase lasts until the 6^{th} week of gestation and involves the formation of bronchopulmonary segments). This suggests that the gestational age of the preterm infant

at the time of birth governs the degree of pulmonary immaturity. Fig 1-1 illustrates the pulmonary development as a function of gestational age.

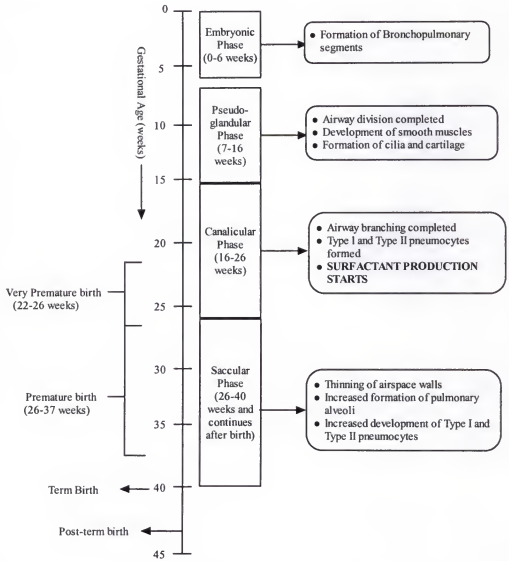


Fig 1-1: Different Stages of Pulmonary Development as a Function of Gestational Age.

The immaturity of the lung necessitates the use of mechanical ventilators to provide artificial respiratory support to the preterm infant. This use of mechanical ventilation leads to significant damage of an already fragile immature lung. Clark et al. (3) have shown that the mechanical damage caused to the immature lungs by mechanical

ventilators leads to fluid and protein leak in the airways, inhibition of surfactant production and increase in pulmonary inflammation. This pulmonary damage predisposes the preterm infant to a wide array of pulmonary complications such as apnea (interruption in breathing), respiratory distress syndrome (pulmonary complication due to insufficient surfactant production) and chronic lung disease (CLD). In addition to the pulmonary complications, the preterm infant also suffers from other physiological complications of premature birth. These include intraventricular hemorrhage (bleeding in the brain which eventually fills up the ventricles leading to brain damage); patent ductus arteriosus (failure of closure of ductus arteriosus leading to heart failure and lack of oxygen to the heart); and retinopathy of prematurity (abnormal growth of blood vessels in the eyes leading to scar formation that can damage the retina).

Pathogenesis of Chronic Lung Disease

Despite significant advances in perinatal and neonatal care, CLD (also known as bronchopulmonary dysplasia) persists as one of the major complications in premature infants who require prolonged mechanical ventilation. Northway et al. (4) have described the occurrence of bronchopulmonary dysplasia as a result of prolong mechanical ventilation. The clinical definition of CLD varies among different healthcare settings. However, the two most commonly accepted definitions in neonatal intensive care units (NICU) are 1) mechanical ventilation and dependence on supplemental oxygen at 28 days postnatal age and 2) the same features at 36 weeks postmenstrual age. The incidence of chronic lung disease among ventilated infants is estimated to be between 4 and 40 % depending on the gestational age; but the highest incidence (in excess of 70 %) occurs in infants weighing less than 1000 g at birth (5). Moreover, the increasing survival of very

immature infants due to significant advancements in neonatal care made in recent years has dramatically increased the number of infants at a risk for developing CLD (6).

An increasing body of evidence suggests that exposure to mechanical ventilation triggers a cascade of inflammatory responses that play a key role in the pathogenesis of CLD in preterm infants (7). A number of factors such as barotraumas induced by mechanical ventilation and production of oxygen-derived free radicals result in the release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF- α), interlukin 6 (IL-6) and interlukin 8 (IL-8). Dooy et al. (8) have shown that lung damage in premature infants may be caused by the failure to downregulate this inflammatory response. Consequently, the discordance between high concentrations of pro-inflammatory mediators and the inability of the premature infant to generate a sufficient anti-inflammatory response makes the premature infant very susceptible to the development of CLD.

The impact of CLD on both mortality and morbidity has made it imperative to develop and implement treatment strategies aimed at preventing/treating CLD. The recognition of a strong correlation between pulmonary inflammation and the development of CLD has resulted in clinical intervention with anti-inflammatory agents. The rationale for using these agents is the modulation of the inflammatory process in the lung thereby reducing the incidence or severity of CLD. Currently, systemic corticosteroids, because of their strong anti-inflammatory properties, appear to be suitable therapeutic agents for the treatment/prevention of CLD. Fig 1-2 shows the various risk factors responsible for preterm birth and eventual development of CLD; and the

beneficial and adverse effects of using systemic corticosteroids, the most widely accepted clinical intervention in CLD.

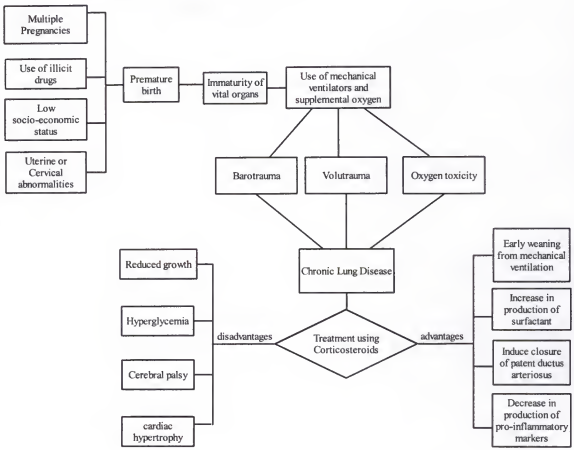


Fig 1-2: Schematic Representation of Development and Treatment of CLD.

Corticosteroids in Chronic Lung Disease

As previously noted, the scientific rationale for using systemic corticosteroid therapy is the reduction in pulmonary inflammation which is considered to play a pivotal role in the onset of CLD (7). Corticosteroids reduce the polymorphonuclear induction in the cells, reduce the production of pro-inflammatory cytokines such as leukotrienes and TNF and induce the closure of patent ductus arteriosus (9). Corticosteroids also enhance the production of surfactant and antioxidant enzymes, decrease bronchospasm, pulmonary and bronchial edema thereby improving the pulmonary compliance in preterm

infants (10, 11). These beneficial effects of corticosteroids facilitate the faster weaning of preterm infants from mechanical ventilators and reduce the duration of supplemental oxygen, factors that are highly implicated in the development of CLD.

Liggins and Howie introduced the concept of using antenatal (steroids administered to the mother at the risk of delivering preterm) corticosteroids for the enhancement of fetal lung maturation (12). They showed that the administration of antenatal corticosteroids to enhance fetal lung maturation resulted in a significant reduction in the incidence of respiratory distress syndrome (RDS) in preterm infants. The landmark study by Liggins and Howie paved the way for a plethora of randomized clinical trials (RCT) that investigated the efficacy of antenatal and postnatal corticosteroids to reduce/prevent the occurrence of CLD. Mammel et al. (13) and Schick et al. (14) showed short-term improvement in pulmonary function and faster weaning from the mechanical ventilator in preterm infants treated with dexamethasone, a potent corticosteroid. Avery et al. (15) showed that in infants treated with dexamethasone, there was significant facilitation in weaning from mechanical ventilators, however, there were no significant differences in the length of hospital stay. Halliday et al. (16) showed the beneficial effects of corticosteroids on lung function leading to earlier extubation of premature infants. Yeh et al. (17) reported that early (< 12 h) postnatal dexamethasone therapy facilitated removal of the endotracheal tube and minimized lung injury in premature infants with severe RDS. Canterino et al. (18) evaluated the effect of antenatal steroid treatment on the development of neonatal periventricular leukomalacia. It was shown that antenatal steroid treatment led to over 50 % decrease in the incidence of periventricular leukomalacia in preterm neonates.

The National Institute of Health (NIH) issued a consensus statement in the spring of 1994 on the multiple benefits of administering a single dose of antenatal steroids for fetal maturation (19). The panel concluded that administration of antenatal corticosteroids to pregnant women at a risk of preterm delivery reduces the incidence of RDS and neonatal mortality. Evidence in the literature was sufficient to advocate the use of antenatal corticosteroids (dexamethasone/betamethasone) up to 7 days before delivery. However, the continuation of corticosteroid therapy beyond 7 days and the advantages/disadvantages of multiple administration of systemic corticosteroids were topics that warranted further research. In the spring of 2000, the NIH again issued clinical recommendations regarding antenatal corticosteroid therapy that entailed giving a single dose of corticosteroids to all pregnant women at 24-34 weeks of gestation who are at a risk of preterm delivery within 7 days (20). However, the report concluded with a cautionary note: "Because of insufficient scientific data from randomized clinical trials regarding the efficacy and safety of repeated courses of corticosteroids, such therapy should not be used routinely. In general, it should be reserved for patients enrolled in randomized controlled trials" (20).

Adverse Effects of Corticosteroids in Preterm Infants

In addition to recognizing the beneficial effects of corticosteroids, the NIH consensus statement also noted the occurrence of serious adverse effects of using systemic corticosteroids in preterm infants. This occurrence of adverse effects after systemic corticosteroid therapy had been shown as early as 1972 by Baden et al. (21) who studied the effect of two doses of hydrocortisone on the incidence of RDS. Follow up studies of surviving premature infants from this trial revealed increased risk of intraventricular hemorrhage (22). Ewerbech and Helwig also reported an increased risk

of intraventricular hemorrhage after using prednisolone in 10 premature infants with severe RDS (23). Fitzhardinge and co-workers (24) followed the trial conducted by Baden et al. (21) and showed that infants who received systemic corticosteroid therapy had increased incidences of neurological complications and lowered motor development. Nevertheless, the attainment of immediate beneficial effects in premature infants after systemic corticosteroid administration led to its unfortunate and indiscriminate use in the 1980s and 1990s despite the early alarming indications of adverse effects and without sufficient establishment of the benefit/risk ratio.

The last two decades have witnessed an alarming increase in the nature and degree of clinical complications observed in preterm infants who are treated with systemic corticosteroids. The degree of adverse effects is highly dependent on the gestational age of the preterm infant (which determines the degree of prematurity), and the degree of development of vital organs and drug transport systems. Many studies have shown the adverse effects after single and multiple doses of systemic corticosteroids. Table 1-1 shows the adverse effects associated with different vital organs of the body (25).

Table 1-1: Adverse Effects of Postnatal Corticosteroid Treatment.

Region of the Body	Adverse Effects
Central nervous system	Motor developmental retardation Atrophy of the dendrites Cerebral palsy
Cardiovascular	Hypertension Cardiac hypertrophy Sustained bradycardia
Metabolic and endocrine	Somatic growth failure Hyperglycemia Proteolysis
Respiratory	Pneumothorax

Yeh et al. (26) studied the outcome at 2 year corrected age of infants who participated in a controlled trial of early (< 12 h) dexamethasone therapy for prevention of chronic lung disease. Results of the study advised against the use of corticosteroids because of its adverse effects on neuromotor function and somatic growth. In addition, the preterm infants also showed transient albeit significant adverse effects such as hyperglycemia, hypertension. Papile et al. (27) conducted a randomized clinical trial to determine the efficacy of early *vs* late dexamethasone therapy in infants at a risk of CLD and reported a decrease in head growth in infants who were receiving dexamethasone. Stark et al. (28) studied the adverse effects of early dexamethasone treatment in extremely low-birth weight (501-1000 g) infants who received mechanical ventilation within 12 h after birth and were randomized to receive either placebo or dexamethasone. Results of the study showed that treatment with dexamethasone was associated with gastrointestinal perforation and decreased growth. In addition, alarming reports in the literature document the termination of clinical trials involving postnatal corticosteroids because of short-term adverse events, including gastrointestinal hemorrhage and intestinal perforation requiring surgery (29). Murphy et al. (30) reported impaired cerebral gray matter growth after treatment of premature infants with dexamethasone. Israel et al. (31) showed in a retrospective study that prolonged treatment of premature infants suffering from chronic lung disease with dexamethasone was associated with hypertrophic cardiomyopathy.

Adverse effects of corticosteroids on the brain have also led to long-term neurological complications. This concern has been amplified by two studies that show significantly more infants with cerebral palsy (32) and reduced neuromotor function (26)

in corticosteroid-treated groups. In addition to the brain-related adverse effects, a variety of adverse effects such as adrenal suppression, immune suppression, bradycardia, weight loss and hyperglycemia have been reported (9). All this information gleaned from a wide variety of scientific literature strongly suggests the adverse effects observed in preterm infants after antenatal and postnatal systemic corticosteroid use. Consequently, the observance of short- and long- term adverse effects, especially on the brain, has simmered the enthusiasm for using corticosteroids systemically for the treatment and prevention of CLD in preterm infants.

P-glycoprotein Transporters and Blood Brain Barrier

As previously mentioned, the systemic administration of corticosteroids results in a variety of neurotoxic adverse effects in preterm infants. This can be due to the enhanced permeability of the corticosteroid across an immature blood brain barrier. The immaturity of the blood brain barrier also leads to incomplete development of efflux systems such as P-glycoprotein transporters.

P-glycoprotein (P-gp, MW 170 KDa) is a 128 amino acid transmembrane glycoprotein and belongs to the family of ATP binding cassette (ABC) transporter proteins. It is highly concentrated on the apical membrane of the endothelial cells of the brain capillaries. It was originally identified because of it's ability to confer multi drug resistance (development of resistance by cancerous cells against a variety of drugs) in mammalian tumor cells (33). The efflux transporters present on the blood brain barrier actively extrude a wide variety of structurally unrelated substrates such as ivermectin, dexamethasone, vinblastin, digoxin, loperimide, domperideone, phenytoin, and cyclosporine A (34, 35). Fig 1-3 schematically represents the blood brain barrier and selected transport mechanisms.

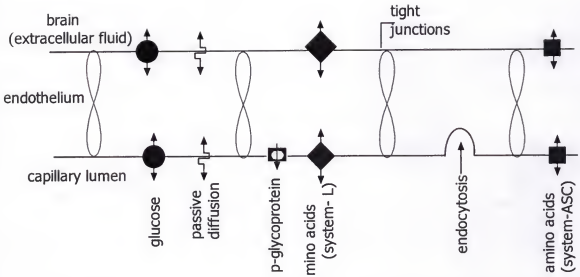


Fig 1-3: Schematic representation of the blood brain barrier and selected transport mechanisms. The arrows indicate the direction of transport (taken from (36)).

The clinical implications of poor development of the p-glycoprotein transporters (due to an immature blood brain barrier) have been previously shown by a number of research groups. Smit et al. (37) have shown that the absence or pharmacological blocking of placental p-gp profoundly increases fetal drug exposure. Lankas et al. (38) have shown that the placental mdr1a p-gp in mice is present in the fetus derived epithelial cells and constitutes a barrier between the fetal and maternal blood circulation. Kalken et al. (39) have studied the expression of p-gp transporters in human tissues at different developmental stages using immunohistochemistry. They did not observe any staining of the embryonic and fetal brain cells upto 28 weeks of gestation. This strongly indicates the absence/poor development of p-gp transporters on the blood brain barrier in preterm infants.

In sharp contrast, the permeability of systemically administered corticosteroids across the blood brain barrier in adults is severely restricted. Dekloet et al. (40) have

shown that the permeability of dexamethasone, a systemic corticosteroid, is restricted across the blood brain barrier of adult rats due to active efflux by the p-gp pump. In addition, Talton et al.(41) and Wang et al. (42), using *ex vivo* receptor binding assay, have evaluated the brain receptor occupancy after intravenous administration of two widely used inhaled corticosteroids, fluticasone propionate (FP) and beclomethasone monopropionate (BMP). Fig 1-4 shows the plot of percent free receptors as a function of time after intravenous administration of FP and BMP.

A

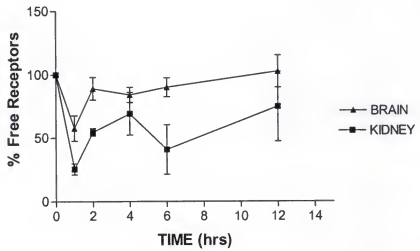


Fig 1-4: Brain and kidney receptor occupancy in rats after intravenous administration (100 µg/kg) of (A) fluticasone propionate (B) beclomethasone monopropionate. Data taken from references (41) and (42) respectively.

B

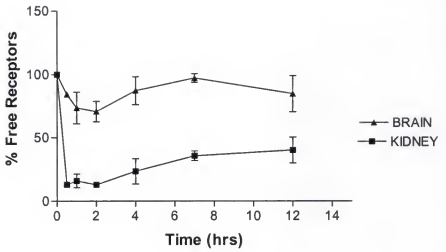


Fig 1-4: Continued

The results clearly show that the permeability of inhaled corticosteroids is severely restricted in adult rats (as indicated by minimal brain receptor occupancy). Chapter 2 provides a detailed evaluation of the role played by p-gp transporters in modulating the brain permeability of inhaled corticosteroids.

Inhaled Corticosteroids In Chronic Lung Disease

Systemic corticosteroids have established profiles of beneficial effects and adverse effects. This leads to the rational question that "how can treatment strategies with systemic corticosteroids be optimized with respect to dose administered, timing of intervention with corticosteroids after birth or perhaps by changing the route of drug administration so that the beneficial effects of the corticosteroids can be maximized and the adverse effects (from high systemic exposure) can be minimized?"

The review of literature shows that a consensus is lacking on the dose that can be used for the treatment or prevention of CLD. Yeh et al. (17) used 1 mg/kg/day of dexamethasone for 3 days and then tapered the dose for 12 days. Stark et al. (43) used

dexamethasone within 24 h after birth (0.15 mg/kg/day) for 3 days and tapered it off over 7 days. O' Shea and colleagues (32) used 0.5 mg/kg/day of dexamethasone and tapered the dose over 42 days. The consensus on optimal dosing schedule is also lacking. Cole and Fiascone (44) have shown that early use (< 2 weeks age) of systemic steroids leads to reduction in CLD and mortality. They also showed that very early use (< 3 days of age) elevates the risk of gastrointestinal complications. However, both of the schedules had adverse effects.

An important parameter that can be modulated to increase beneficial effects and decrease systemic exposure to corticosteroids is the route of drug administration. Delivery of corticosteroids through the inhalation route is a plausible alternative. Major advantages of delivering drugs through the inhalation route include direct delivery of the drug to the site of inflammation (i.e., the lungs), rapid onset of action, lower doses needed for effective therapy leading to less spill-over into the systemic circulation and accessibility to systemic circulation without traversing the liver (particularly suitable for drugs that are systemically active but show a high first pass effect after oral administration) by absorption across the pulmonary epithelium. The advantages of delivering corticosteroids through the inhalation route for treating pulmonary inflammatory disorders such as asthma have been clearly established. As the use of corticosteroids to counteract the inflammatory reaction in the lung is the common denominator between asthma and CLD, it can be expected that administering corticosteroids through the pulmonary route to premature infants suffering from CLD will result in a higher benefit/risk ratio.

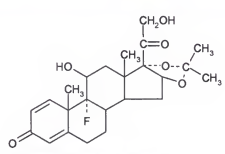
A number of research groups have investigated the benefits of delivering a variety of corticosteroids to premature infants through the pulmonary route. Arnon et al. (45) studied the clinical efficacy of budesonide (600 μg twice daily) *vs* placebo administered by metered dose inhaler and spacer directly into the endotracheal tube of intubated infants. Results showed a significant reduction in the need for mechanical ventilation in the budesonide-treated group without concurrent adverse effects. Jonsson et al. (46) showed that budesonide aerosol delivered through a dosimetric jet nebulizer decreased the requirement for mechanical ventilation without significant adverse effects in premature infants who were at a high risk for developing CLD.

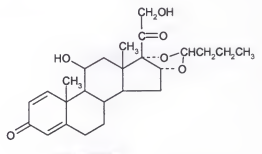
On the other hand, a number of studies have shown the limited effectiveness of inhaled glucocorticoid therapy in premature infants suffering from CLD. Groneck et al. (47) did not observe any reduction in tracheal inflammatory markers after 10 days of inhaled beclomethasone therapy (500 μg tid) initiated on day 3 of life in ventilated infants compared to rapid reduction in tracheal inflammatory markers after 3 days of systemic dexamethasone therapy (0.5 mg/kg/day). Dimitriou et al. (48) investigated the degree and onset of the clinical response and adverse effects observed after a 10 day course of either systemically administered dexamethasone (0.5 mg/kg/day) or nebulized budesonide (100 μg qid) in a randomized trial of 40 preterm infants who required mechanical ventilation after 5 days or supplemental oxygen for at least 14 days. Results indicated a greater and faster onset of action after systemic administration of dexamethasone. Inwald et al. (49) have previously shown elevated levels of chemokines in the broncho-alveolar lavage (BAL) fluid of infants treated for respiratory distress syndrome (RDS). To study the effect of inhaled budesonide in reducing the levels of

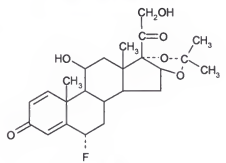
chemokines, Inwald et al. (50) measured the levels of chemokines in 12 preterm infants who were ventilated for RDS. No significant changes in the levels of chemokines were found in the inhaled budesonide group. Cole et al. (51) conducted a multicenter trial to determine if inhaled beclomethasone dipropionate in premature infants (< 33 weeks of gestation) would reduce the frequency of bronchopulmonary dysplasia (BPD). Results showed a similar frequency of BPD in the beclomethasone and placebo treated groups; however, fewer infants in the inhaled beclomethasone therapy group required additional systemic corticosteroids or mechanical ventilation.

One should be very cautious in interpreting the limited success of inhalation therapy in premature infants. Results should be analyzed in light of the various complexities associated with delivering aerosolized medication to preterm infants. The clinical efficacy of aerosolized corticosteroids for treatment of pulmonary disorders such as CLD is contingent on stringent control of a variety of factors. These factors include amount of dose deposited in the lungs, particle size and regional distribution of the deposited dose in the lung, and device used to deliver the dose. In addition to these factors, patient-related factors such as degree of lung development influence the clinical efficacy of inhaled formulations. Further, the highly lipophilic nature of the corticosteroids in conjugation with high absorptive surface provided by the pulmonary epithelium results in rapid absorption of most commercially available glucocorticoids such as flunisolide, triamcinolone acetonide, beclomethasone dipropionate and budesonide (Fig 1-5). This rapid absorption from the lungs into the systemic circulation results in low pulmonary residence time (the time for which the drug stays in the lung

before being absorbed into the systemic circulation) leading to very low pharmacologically active pulmonary corticosteroid concentrations.







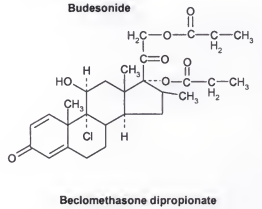


Fig 1-5: Chemical Structures of Some Commonly Used Inhaled Corticosteroids.

Hochhaus et al. (52), through a series of simulations, have shown that inhaling a glucocorticoid solution does not necessarily result in pulmonary targeting because a solution is rapidly absorbed from the lung into systemic circulation. This leads to adverse systemic effects and a lower benefit-to-risk ratio. Hence, alternative strategies for drug delivery are urgently required that will increase the pulmonary selectivity of the drug. This will help in achieving increased pulmonary targeting and reduction in undesired systemic effects thereby leading to a higher benefit-to-risk ratio.

Strategies for Improving Pulmonary Selectivity

The ultimate goal of achieving pulmonary selectivity is a reduction in dose required to produce the desired beneficial effects with concomitant reduction in adverse effects.

For all forms of pulmonary administration, only a small portion of the drug is delivered to the lungs whereas the major part of the drug is deposited in the oropharynx and consequently swallowed. The portion of the drug reaching the lungs is either rapidly absorbed into systemic circulation or removed from the upper portions of the airways by mucociliary transporters. The swallowed portion of the drug, depending on the oral bioavailability, enters the systemic circulation where it can show systemic adverse effects. Hence, the efficient removal of this systemically available drug (which is a combination of drug coming from the lungs and the drug that is orally absorbed) is pivotal for achieving pulmonary selectivity. It has been shown that a variety of local and systemic factors are involved in achieving pulmonary selectivity (53). Table 1-2 lists the factors important for achieving pulmonary targeting

Table 1-2: Factors for Achieving Pulmonary Targeting.

Pulmonary Components	Systemic Components
Efficiency of pulmonary deposition Pulmonary residence time Pulmonary absorption rate Pharmacodynamic drug characteristics in the lung	Oral bioavailability Clearance Volume of distribution

As indicated in the table, one of the factors that plays a key role in determining pulmonary selectivity is the pulmonary residence time. A variety of approaches can be adopted to increase the pulmonary residence time of the drug. These approaches include: 1) slow dissolution rate of the drug particles, 2) corticosteroid esterification (in case of budesonide) and 3) use of slow release systems such as liposomes and nano-coatings.

The data available for inhaled corticosteroids suggests that drugs with slower dissolution rate such as fluticasone propionate show higher pulmonary targeting (54). In

addition, using an animal model for pulmonary targeting, it was shown that the degree of pulmonary targeting of intratracheally administered TA increased from solutions to micronized particles to crystal suspensions (55, 56).

Recent biochemical studies have shown that budesonide, a widely used inhaled corticoseroid, is intracellularly esterified (57, 58). These esters are unable to traverse the pulmonary membranes and are trapped as inactive "pro-drugs". The esters are eventually cleaved by esterases present in the lung thereby releasing the active drug. Although this is a novel mechanism to increase the pulmonary residence time, more studies are required to determine whether the drug being "trapped" as ester represents a clinically relevant portion of the dose.

The use of sustained release systems has gained widespread attention during the last two decades. Better control over the rate of drug release, less frequent drug administration, improvement in patient compliance and reduction in the fluctuation of plasma levels are some of the factors that have led to the successful adoption of sustained-release drug-delivery systems in a variety of therapeutic areas (such as ophthalmic, transdermal etc.). In addition to the advantages previously mentioned, a major advantage of sustained-release formulation is retention of the drug in the local area for a longer period of time. This leads to an increase in the local effects and significant reduction of systemic exposure. In contrast, conventional dosage forms provide immediate release of the drug that necessitates frequent dosing for maintenance of therapeutic levels.

As previously noted, the enthusiasm of using systemic corticosteroids in preterm infants suffering from chronic lung disease has simmered due to high incidence of

systemic adverse effects. Hence, the pulmonary delivery of sustained release formulations of inhaled corticosteroids are expected to exhibit higher pulmonary residence time thereby leading to significant improvement in pulmonary selectivity. Appropriate modifications of the drug/delivery system can potentially result in a wide spectrum of sustained release formulations that markedly differ in their pharmacokinetic/ pharmacodynamic properties when compared with the conventional form of the drug.

Sustained Release Drug Delivery Systems

Liposomal and microencapsulated (polymer coated) formulations such as microspheres have gained widespread attention for their ability to provide sustained release of the encapsulated drug. Next is a brief description, including pharmaceutical applications and potential limitations.

Liposomes

Liposomes have evolved into a major class of drug-delivery systems since their discovery by Bangham et al. (59). Liposomes are microscopic vesicles composed of multilamellar phospholipid bilayers alternating with hydrophilic compartments. The drug, depending on its physicochemical characteristics, is either incorporated in the aqueous or the lipid bilayer. The size (diameter) of the liposomal formulation varies from 20 nm to 20 μm. The ability of the liposomes to modulate the pharmacokinetics and biodistribution of the encapsulated drug has provided impetus for their use in a number of medical complications (such as cancer, fungal infections etc.). A number of commercially available liposome based products such as Doxil™ (doxorubicin) and ambisome™ (amphotericin B) have obtained FDA approval and are being routinely used (60).

The advantages of using liposomes in inhalation therapy have been well documented (61). In addition to acting as a drug reservoir in the lungs, liposomes also facilitate the achievement of high concentrations of the drug in the infected macrophages. The similarity between the phospholipids used for preparation of liposomes and the naturally occurring phospholipids (which form the surfactant system in the lungs) minimizes the incidence of toxicity. Optimally designed liposome-based drug-delivery systems can potentially prolong the pulmonary residence time of the drug and lead to significant decrease in systemic exposure.

Rothi et al. (62) and Hochhaus et al. (63) have explored the use of triamcinolone acetonide phosphate (TAP) as pulmonary targeted drug-delivery systems . They showed that intratracheal administration of liposome encapsulated TAP provided sustained receptor occupancy and improved pulmonary targeting in comparison to TAP solution. Other groups have also reiterated the beneficial effects of encapsulating drugs in liposome to increase the pulmonary retention time. For example, Brattsand and co-workers (64) demonstrated that budesonide palmitate liposomes, but not budesonide, showed improved pulmonary targeting in a rat alveolar model of pulmonary inflammation. Although the various formulation parameters such as choice of lipids, incorporation of cholesterol (for decreasing the permeability of the bilayer to avoid leakage etc.) influence the liposomal characteristics such as size, encapsulation efficiency, the route of administration of the liposomal formulation ultimately determines the PK/PD profile of the entrapped drug.

The liposomal encapsulated formulations also have potential limitations such as low shelf life stability, leakage of the encapsulated drug, inability to permeate capillary

endothelial cells in the intact form, and low encapsulation efficiencies with hydrophilic drugs.

Microencapsulation

Microencapsulation is a technique of applying thin coatings to small solid particles. The basic parameters that need to be taken into account while designing microencapsulated formulations include the core (i.e., the active drug), the coating material (which to a large extent governs the physical and chemical properties of the microencapsulated formulation), and the method used to microencapsulate the drug. Flexibility in the choice of core material (solid particles or dispersed material) has significantly contributed toward improving formulation acceptability (e.g., taste masking, in the case of acetaminophen; reduction of gastric irritation from potassium chloride; and stability toward oxidation for vitamin A palmitate) (65). The choice of the coating material is, in part, contingent on the nature of the drug to be encapsulated, as the coating material should be nonreactive and compatible with the active drug. In addition, the use of biodegradable polymers such as poly (l-lactic acid (PLA) and poly (lactic-co-glycolic acid) (PLGA) as coating material has also gained popularity because of the easy bio-degradation of these polymers by *in vivo* enzymatic hydrolysis.

Microspheres

Microspeheres have gained widespread importance as pulmonary drug delivery systems due to several advantages such as higher shelf life and longer *in vivo* retention of the drug as compared to liposomes. Respirable PLGA micropsheres of rifampin have been shown to reduce the incidence of inflammation and lung damage in a guinea pig lung infection model(66). Kawashima et al. (67)have shown the utility of pulmonary delivered insulin with nubulized PLGA microspheres to prolong the hypoglycemic effect.

PLGA microspheres of isoproterenol have been shown to reduce bronchoconsriction(68). The coating of biodegradable polymers are mainly applied using spray drying technology which significantly increases the polymer load. This has led to development of alternate methods of coating the drug, which can reduce the polymer load on the drug particles.

One way to coat the drug is by using pulse laser deposition (PLD), a novel laser based technique. The method essentially involves the deposition of ultra thin coatings (10-1000 nanometers) of biodegradable polymers on the drug particles that are typically in the size range of 1-5 μm. This results in an extremely low polymer load (generally less than 1% by mass) (69). Fig 1-6 provides a schematic diagram of the PLD set up which is used to deposit polymeric coatings on drug particles.

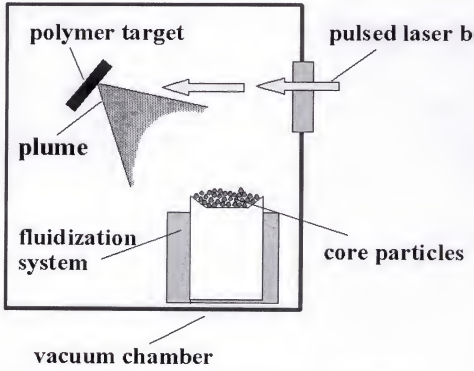


Fig 1-6: Schematic Diagram of the PLD set up (taken from reference (41).

Briefly, the coating procedure consists of a biodegradable polymer target and a fluidized bed of drug particles. The laser beam enters the vacuum chamber and ablates the polymer target that forms the plume. The plume is consequently deposited on the

fluidized drug particles. Various factors such as choice of the polymer, coating time can be optimized to obtain sustained release formulations. The microencapsulated formulation thus obtained is expected to sustain the release of the drug powder, thereby leading to higher pulmonary residence time and improved pulmonary targeting.

The *in vivo* efficacy of microencapsulated (using PLD) corticosteroids has been shown in adult rats by Talton et al. (41). It was shown that microencapsulated budesonide dry powder exhibited slower pulmonary absorption and significant increase in pulmonary targeting as compared to the free powders of budesonide in adult rats. Assuming the applicability of these results to the neonatal rat model, higher pulmonary targeting can be expected after pulmonary delivery of microencapsulated corticosteroids.

Objectives

The following specific aims were tested:

- To study the role played by p-gp transporters in modulating the brain permeability of inhaled corticosteriods in mice.
- To determine the pulmonary targeting and investigate the potential reasons for differences in brain receptor occupancies between neonatal and adult rats after intratracheal instillation of triamcinolone acetonide phosphate (TAP) solution.
- To determine whether intratracheal instillation of poly (l-lactic acid) (PLA) encapsulated budesonide demonstrates pulmonary targeting in the neonatal rat model.

CHAPTER 2
ROLE OF P-GLYCOPROTEIN TRANSPORTERS IN MODULATING THE BRAIN PERMEABILITY OF INHALED CORTICOSTEROIDS

Introduction

The blood brain barrier (BBB) restricts the entry of a variety of therapeutically active agents from the systemic circulation into the central nervous system (CNS). The endothelial cells of the brain capillaries, connected *via* tight junctions, form a physical barrier and limit the penetration of hydrophilic substrates. In addition, the efflux transporters present on the BBB actively extrude a wide variety of structurally unrelated substrates such as ivermectin, vinblastin, digoxin, loperimide, domperideone, phenytoin, and cyclosporine A (34, 35). This active extrusion by the efflux pumps has severely limited the clinical efficacy of therapeutic moieties used for treating brain cancer (70) and HIV infections in the brain (71). As previously mentioned, P-glycoprotein (P-gp) plays a very critical role in regulating the movement of xenobiotics across the blood brain barrier.

The availability of knockout mice has proven to be a major tool to investigate the role of p-glycoprotein transporters in modulating the permeability of drugs across the BBB (72). Using this model, Schinkel et al. (72) have shown that the levels of ivermectin in the brain of mdr1a (-/-) increased about 90 fold as compared to wild type mice . Mayer et al. (73) showed that digoxin accumulated in the brain of mdr1a (-/-) mice, which was in sharp contrast to very low levels in wild type mice. Schinkel et al. (35) showed a sevenfold and fourfold increase in the levels of loperamide and

ondansetron respectively in mdr1a (-/-) than wild type mice. These results clearly demonstrate the pivotal role played by P-gp in modulating the permeability of drugs across the blood brain barrier and show that the presence or absence of p-gp on the blood brain barrier can either restrict the permeability or lead to significantly elevated levels of the P-gp substrates.

The primary focus of our work was to evaluate whether p-gp transporters influence the permeability of triamcinolone acetonide phosphate (TAP), one of the clinically relevant inhaled glucocorticoid. A previously developed (55) *ex vivo* receptor binding assay was used to monitor the free cytosolic receptors in the brain and liver of wild type and knockout mice after intravenous administration of 100 µg/kg TAP. This assay was used because it is a surrogate marker of pharmacologically relevant free drug concentrations in different tissues.

Hypothesis

We expect to see significantly higher brain receptor occupancy in mdr 1a mice due to absence of p-gp transporters. To test our hypothesis, the brain and liver receptor occupancies were monitored in wild type and mdr1a mice after intravenous administration of TAP (100 µg/ kg).

Materials and Methods

Triamcinolone acetonide phosphate solution (TAP) (54.4 mg/mL) was obtained from Bristol Myers Squibb (BMS), Munich, Germany. ({6,7-^{3}H} triamcinolone acetonide, 38 Ci/mmol) was obtained from New England Nuclear (Wilmington, DE). All other unlabelled chemicals were obtained from Sigma (St. Louis, MO) or equivalent sources.

Preparation of Drug and Radiolabelled Solutions

TAP solution (54.4 mg/mL) was diluted with PBS to obtain a final concentration of 50 μg/mL. Suitable volume of this solution (equivalent to 100 μg/ kg) was injected into the mice through the tail vein. 20 nM ^{3}H labeled TA (prepared in the incubation buffer) and a mixture of 20 nM ^{3}H labeled TA and 20 μM unlabelled TA was used to determine the total and non- specific binding respectively.

Animal Procedures

All animal procedures were approved by the Institutional animal care and use (IACUC), University of Florida, an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved facility. Wild type mice and mdr1a knockout mice (30 ± 5 g) were obtained from Taconic (Germantown, NY) and were housed in sterile pathogen free (SPF) environment. The animals were housed in the operating room 12 h before the experiment to accustom them to the new environment. On the day of the experiment, the mice were gently handled (to produce minimum stress) and weighed. The mice were anesthetized with an anesthetic mixture (1.5 ml of 10 % v/v ketamine, 1.5 ml of 2 % v/v xylazine and 0.5 ml of 1 % v/v acepromazine) at the dose 1 ml/kg. The depth of anesthesia was checked using tail pinch or pedal withdrawal reflex. Once the mice were under complete anesthesia, either 100-125 μL of glucocorticoid (TAP) solution or saline (for placebo) was slowly injected into the tail vein using a tuberculin syringe with a 27-guage needle. The mice were decapitated at 1, 2.5 and 6 hours after tail vein injection of the glucocorticoid drug solution. The brain and liver were removed and immediately processed for receptor binding studies.

Ex Vivo Receptor Binding Assay

A previously developed *ex-vivo* receptor binding assay was used (63). Immediately after decapitation, the brain and liver were resected and placed on ice. The weighed tissue was added to 10 times (for liver) and 4 times (for brain) organ weight of ice-cold incubation buffer (10 mM Tris/HCl, 10 mM sodium molybdate, 2 mM 1,4-dithioerythritol). 2 mL of the homogenate was incubated with 5 % charcoal (in distilled water) for 10 minutes to remove endogenous corticosteroids. The homogenate was centrifuged for 20 min at 20,000 g at 4° C in a Beckman centrifuge equipped with a JA-21 rotor to obtain a clear supernatant. Since the amount of cytosol obtained from various tissues of mice was very less, for all mice experiments, the volume of cytosol used, the volume of tracer added, the volume of charcoal added to remove excess radioactivity and the supernatant collected for reading in the scintillation counter were reduced to half of the volumes used for the rat experiments.

Aliquots of the supernatant (75 µL) were added to pre-chilled microcentrifuge tubes containing 25 µL of 20 nM ^{3}H labeled TA or a mixture of 20 nM ^{3}H labeled TA and 20 µM of unlabelled TA to determine the total binding and the non-specific binding respectively. The microcentrifuge tubes were vortexed and incubated at 4 ° C for 18 h.

After the incubation, 100 µL of activated charcoal (5 % in water) was added to the microcentrifuge tubes to remove excess radioactivity. The microcentrifuge tubes were vortexed, centrifuged for 5 minutes and 125 µL of supernatant was removed and added to the scintillation vial. 2.5 mL of the scintillation cocktail (Cytoscint™, ICN Biomed, Costa Mesa, CA) was added and the scintillation vials were read in a scintillation counter (Beckman, LS 5000 TD, Palo Alto, CA) to obtain the radioactive counts (measured in disintegrations per minute (dpm's)) in different tissues.

For a given tissue (liver or brain), the radioactivity counts corresponded to the total binding (specific + non specific) of the tracer. The dpm's corresponding to the non-specific binding (obtained by incubating the cytosol with a high concentration of a mixture of 20 nM ^{3}H labeled TA and 20 μM unlabelled TA for mice experiments, removing excess radioactivity and determining the radioactive counts in the supernatant) was subtracted from the total binding to obtain estimates of the specific binding. The specific binding obtained in the rats administered saline (placebo) corresponded to 100 % free receptors. The % free receptors present in the brain or liver was calculated as

$$\% \text{ free receptors in a tissue} = \frac{\textit{specific binding in a tissue of rat administered TAP}}{\textit{specific binding in a tissue of rat administered saline}} * 100$$

For each tissue, the cumulative $AUC_{0\text{-}6h}$ calculated from the % free receptors *vs* time profile was subtracted from 600 (each hour of the experiment corresponds to 100 % free receptors) to obtain the cumulative $AUC_{0\text{-}6h}$ for % bound receptors. The average receptor occupancies (AUC) in the brain and liver for wild type and mdr1a mice were obtained by dividing the cumulative receptor occupancy ($AUC_{0\text{-}6h}$) by 6h (the duration of experiment). The average receptor occupancies observed in each tissue was compared using a student t test.

Results

Fig 2-1 and 2-2 show the % free receptors *vs* time profiles in the liver and brain respectively, after intravenous administration (100 μg/kg) of TAP to wild type and knockout mice. Table 2-1 shows the average AUC estimates obtained in the brain and liver. Intravenous administration of TAP resulted in similar average hepatic AUC in mdr1a and wild type mice (37.8 % *vs* 34.9 %) ($p>0.05$). However, the average brain

AUC in mdr1a deficient mice was significantly higher in knockout mice than wild type mice (47.5 % *vs* 11.5 %) ($p<0.001$).

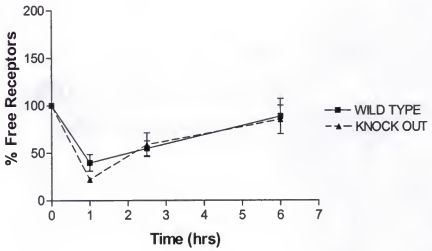


Fig 2-1: Liver Receptor Occupancy in wild type and mdr1a (-/-) mice after intravenous administration (100 μg/kg) of triamcinolone acetonide phosphate.

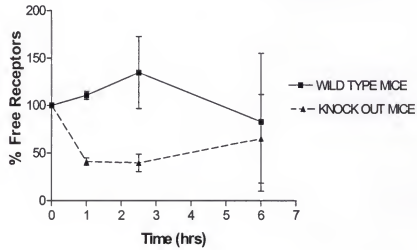


Fig 2-2: Brain Receptor Occupancy in wild type and mdr1a (-/-) mice after intravenous administration (100 μg/kg) of triamcinolone acetonide phosphate.

Table 2-1: Average AUC's in the brain and liver of wild type and mdr1a mice after intravenous administration of TAP.

	Dose (μg/ kg)	Average AUC (%)	
		Brain	Liver
Wild type mice	100	11.5	34.9
mdr1a mice	100	47.5	37.8

Discussion

The blood -brain barrier regulates the composition of extra cellular fluid and protects the brain against changes in the systemic circulation (74). The permeability across the blood brain barrier increases with increasing lipophilicity but decreases again when a maximum lipophilicity is achieved (75). However, the CNS permeability of some lipophilic substances such as vinblastin (Log P=1.7), vincristine (Log P=2.1) is very limited. This can be explained on the basis of the presence of efflux mechanisms which actively efflux the drugs from CNS into the systemic circulation. P-gp is one of the efflux transporters that plays a critical role in modulating the permeability of xenobiotics across the blood brain barrier.

Although corticosteroids are lipophilic and are expected to easily cross the blood brain barrier, the limited amount of available literature clearly shows that penetration of a number of systemically used glucocorticoids such as dexamethasone and prednisolone is modulated by p-gp (76-78). In addition, Talton et al. (41) and Wang et al. (42) have used a previously developed *ex vivo* receptor binding assay (55) to monitor the glucocorticoid receptor occupancy in the brain and kidney after intravenous administration of a majority of clinically relevant inhaled glucocorticoids such as budesonide, fluticasone propioinate,

beclomethasone dipropionate, beclomethasone monopropionate and triamcinolone acetonide. The results from these studies have shown minimal brain receptor occupancy after intravenous/intratracheal administration of inhaled corticosteroids thereby suggesting the the involvement of efflux mechanisms. To establish a clear link between the minimal receptor occupancy in the brain and the active efflux by the p-gp pump, the brain and liver receptor occupancy was monitored in wild type and mdr1a adult mice after intravenous administration of TAP. The average AUC estimates calculated from the % free receptors *vs* time profiles clearly show significantly higher brain receptor occupancy of TAP in knockout mice. This strongly indicates the involvement of p-glycoprotein transporters in the active efflux of corticosteroids from the brain thereby modulating the pharmacologically active concentrations in the brain. The hepatic receptor occupancies were similar for wild type and knock out mice.

Although similar results have been shown by De Kloet et al. (40) for systemic corticosteroids, the assay methodology used and the nature (inhaled *vs* systemic) of corticosteroid used in our study were different. De Kloet et al. measured the total concentrations of subcutaneously administered ^{3}H dexamethasone in mdr1a (-/-) and mdr1a (+/+) mice, whereas we used *ex vivo* receptor binding assays (surrogate marker of free levels) to assess the corticosteroid receptor occupancies in the brain.

The incidence of drug–drug interactions due to modulation of brain permeability of p-gp substrates have been widely reported (79-81). These and similar studies shed light on the changes in disposition of p-gp substrates when co-administered with p-gp modulators. The results of our study suggest that the brain permeability of inhaled glucocorticoids is also modulated by p-gp transporters. Consequently, concomitant

administration of inhaled glucocorticoids and p-gp inducers/inhibitors such as quinidine and verapamil can potentially lead to clinically relevant drug-drug interactions.

In conclusion, the results of our study strongly suggest the critical role played by p-gp in modulating the permeability of inhaled glucocorticoids. The understanding of the important role played by p-gp transporters in modulating the permeability of drugs across the blood brain barrier will significantly contribute towards development of effective medications for CNS related disorders.

Conclusions

- We observed significantly higher brain receptor occupancy in knockout mice than wild type after intravenous administration of TAP. This suggest the extrusion of inhaled corticosteroids by the p-gp transporters thereby preventing brain receptor occupancy.
- These results in conjugation with the minimal brain receptor brain occupancy observed after intravenous administration suggest the pivotal role played by p-gp transporters in reducing pharmacologically relevant free levels of inhaled corticosteroids.
- The involvement of active transport mechanisms in modulating the brain uptake of inhaled corticosteroids argue for the possibility of drug-drug interactions.

CHAPTER 3
ASSESMENT OF PULMONARY TARGETING AND BRAIN PERMEABILITY OF TRIAMCINOLONE ACETONIDE PHOSPHATE, AN INHALED STEROID, IN NEONATAL RATS USING *EX VIVO* RECEPTOR BINDING ASSAY

Introduction

Inhaled corticosteroids are highly lipophilic moieties and are rapidly absorbed across the pulmonary epithelium into the systemic circulation (82, 83). This rapid absorption of the corticosteroids into the systemic circulation may explain the high incidence of adverse effects observed in preterm infants after systemic corticosteroid administration. It has been previously shown that the extent of the pharmacological effects (or side effects) of the glucocorticoid is directly related to the fraction of receptors occupied (84). Hence, tracking corticosteroid receptor occupancy in the local (lungs) and systemic (brain, liver) organs can potentially provide a reasonably accurate assessment of the beneficial effects/side effects of inhaled corticosteroids.

As previously mentioned, the placental p-gp transporters play a very critical role role in protecting the developing fetus against maternal xenobiotic exposure. Consequently, the absence or pharmacological blocking of these transporters results in increased fetal exposure(37, 38).

We used a previously developed *ex vivo* receptor binding assay to simultaneously assess the fraction of receptors occupied in the local (lung) and systemic (liver, brain) organs after intratracheal administration of different doses of triamcinolone acetonide phosphate (TAP). The validity of such a model has been previously established in adult rats by Hochhaus et al. (55) for assessing the pulmonary targeting observed after

intratracheal instillation of TAP solution and liposomal encapsulated TAP. The same model has been utilized for determining the degree of pulmonary targeting in neonatal rats.

Hypothesis

We expect to see similar pulmonary and hepatic receptor occupancies after intratracheal administration of various doses of TAP. Further, we expect to see significant brain receptor occupancy at the higher doses (25 and 50 μg/kg) of TAP. To test our hypothesis, the local (lung) and systemic (liver and brain) receptor occupancies were monitored in neonatal (10-11 days old) rats after intratracheal instillation of TAP at at different doses (2.5, 25 and 50 μg/kg).

Materials and Methods

Triamcinolone acetonide phosphate solution (TAP) (54.4 mg/mL) was obtained from Bristol Myers Squibb (BMS), Munich, Germany. Phosphate buffered saline (pH 7.4) was obtained from Cellegro® (Mediatech, Herndon, VA). ({6,7-^{3}H} triamcinolone acetonide, 38 Ci/mmol) was obtained from New England Nuclear (Wilmington, DE). All other chemicals were obtained from Sigma (St. Louis, MO) or equivalent sources.

Preparation of TAP and Radiolabelled Solution

TAP solution (54.4 mg/mL) was suitably diluted with PBS to obtain 50 μg/mL of working stock solution. Suitable volumes of the working stock solution were intratracheally administered at doses of 2.5, 25 and 50 μg/kg TAP.

10 nM ^{3}H labeled triamcinolone acetonide (TA), prepared in incubation buffer (mixture of 10 mM Tris/HCl and 10 mM sodium molybdate in cold water) was used as tracer solution. A mixture of 10 nM ^{3}H labeled TA and 10 μM unlabelled TA, prepared in incubation buffer, was used to estimate the non-specific binding.

Animal Procedures

All animal procedures were approved by the institutional animal care and use committee, (IACUC), University of Florida , an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved facility. Neonatal rats (10-11 days old) were obtained from Harlan (Indianapolis, Indiana). The rats were anesthetized with an anesthetic mixture (1.5 ml of 10 % ketamine, 1.5 ml of 2 % xylazine and 0.5 ml of 1 % acepromazine) at the dose of 1 ml/kg. The skin on the neck was shaved and the area was cleaned with betadine solution. A 1-cm incision was made in the skin with a sterile scalpel blade to expose the underlying musculature. The muscles were gently teased apart with a sterile curved hemostat to expose the trachea. Silk suture was passed under the trachea for further manipulation. An incision was made between a pair of tracheal rings and either TAP solution (2.5, 25 or 50 μg/kg) or saline (to placebo rats) was administered. Following surgery, animals were placed on a fresh drape overlying a heating pad. The neonatal rats were kept warm with the aid of a heating pad and overhead light and the body temperature was monitored *via* a mouse rectal probe connected to a microprobe thermometer. The neonatal rats were decapitated at various time points (1, 2.5, 4 and 6 h) and the lungs, liver and brain were removed. The weighed tissue was added to 4 times (for lungs and brain) and 10 times (for liver) organ weight of ice cold incubation buffer (10 mM Tris/HCl, 10 mM sodium molybdate, 2 mM 1,4-dithioerythritol). The homogenate was incubated with 5 % charcoal (in distilled water) for 10 minutes to remove endogenous corticosteroids. The homogenate was centrifuged for 20 min at 20,000 X g at 4 °C in a Beckman centrifuge equipped with a JA-21 rotor to obtain a clear supernatant. Aliquots of the supernatant (150 μL) were added to pre-chilled microcentrifuge tubes containing 50 μL of either 10 nM ^{3}H labeled TA for

determining the total binding or a mixture of 10 nM ^{3}H labeled TA and 10 μM of unlabelled TA for determining the non-specific binding. The microcentrifuge tubes were vortexed and incubated at 4 ° C for 18 h.

After the incubation, 200 μL of activated charcoal (5 % in water) was added to the microcentrifuge tubes to remove excess radioactivity. The microcentrifuge tubes were vortexed, centrifuged for 5 minutes and 300 μL of the supernatant was removed and added to the scintillation vial. 5 mL of the scintillation cocktail (Cytoscint™, ICN Biomed, Costa Mesa, CA) was added and the scintillation vials were read in a scintillation counter (Beckman, LS 5000TD, Palo Alto, CA) to obtain the radioactive counts (measured in disintegrations per minute (dpm's)) in different tissues.

For a given tissue (lung, liver or brain), the radioactivity counts (measured in dpm's) correspond to the total binding (specific+ non specific) of the tracer. The dpm's corresponding to the non-specific binding (obtained by incubating the cytosol with a high concentration of unlabelled TA, removing excess radioactivity and determining the radioactive counts in the supernatant) was subtracted from the total binding to obtain estimates of the specific binding. The specific binding obtained in the rats administered saline (placebo) corresponded to 100 % free receptors. The % free receptors present in the lung, liver or brain was calculated as

$$\% \text{ free receptors in a tissue} = \frac{\textit{specific binding in a tissue of rat administered TAP}}{\textit{specific binding in a tissue of rat administered saline}} * 100$$

For each tissue, the cumulative $AUC_{0\text{-}6h}$ calculated from the % free receptors *vs* time profile was subtracted from 600 (each hour of the experiment corresponds to 100 % free receptors) to obtain the cumulative $AUC_{0\text{-}6h}$ for % bound receptors. The average receptor occupancies (AUC) in the lung, liver and brain were obtained by dividing the

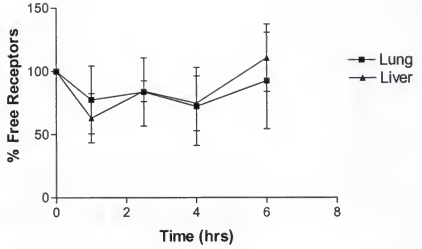
cumulative receptor occupancy ($AUC_{0\text{-}6h}$) by 6h (the duration of experiment). The differences in pulmonary and hepatic receptor occupancies (AUC_{lung}-AUC_{liver}) after different doses were compared using student t test.

Results

Fig 3-1 (A-C), 3-2 (A-C) and 3-3 (A-C) show the plots of % free corticosteroid receptors in the lung, liver and brain of neonatal rats as a function of time after intratracheal administration of different (2.5, 25 and 50 μg/kg) doses of TAP. Fig 3-4 shows the plot of % free receptors as a function of time after intratracheal instillation of 100 μg/ kg of TA. Table 3-1 gives the average area under the curve (AUC) estimates obtained from the plots of % free receptors *vs* time.

After intratracheal administration of 2.5μg/kg TAP to neonatal rats, the average lung, liver and brain receptor occupancies were 18.3 ± 4.5 %, 17.4 ± 13.5 %, -14.7 ± 11.9%. After intratracheal administration of 25 μg/kg TAP, the average lung, liver and brain receptor occupancies were 36.3 ± 12.6 %, 45.3 ± 7.8 % and 45.7 ± 9.7 respectively. However,.after administration of 50 μg/kg TAP, the lung, liver and brain receptor occupancies were 59.9 ± 9.4. %, 50.8 ± 13.0, 47.0 ± 10 respectively. As shown the table, the average AUC estimates in the lung and liver were similar after intratracheal instillation of various doses of TAP. However, the average AUC estimates in the brain were significantly higher at 25 and 50 μg/kg as compared to the lowest dose (2.5 μg/kg).

A



B

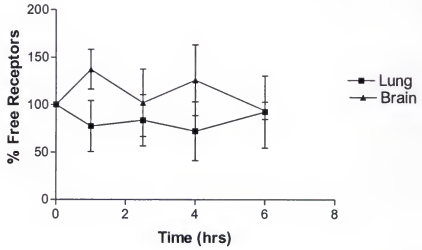


Fig 3-1: Percent free receptors *vs* time profiles in (A) lung *vs* liver (B) lung vs Brain and (C) brain *vs* liver after intratracheal instillation of 2.5 μg/kg of TAP in neonatal rats.

C

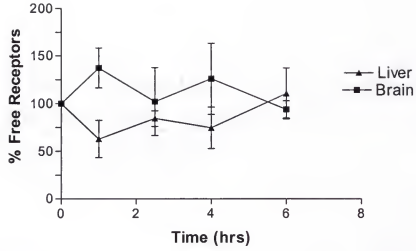


Fig 3-1: Continued

A

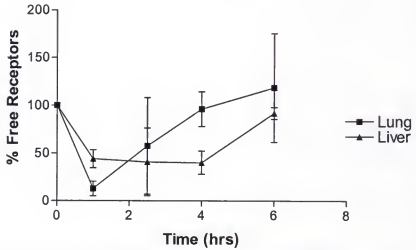
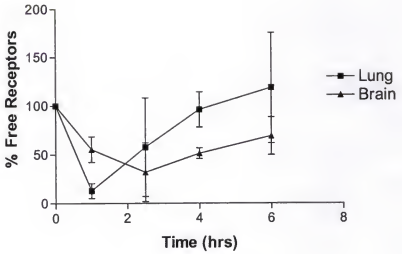


Fig 3-2: Percent free receptors *vs* time profiles in (A) lung *vs* liver (B) lung *vs* brain and (C) brain *vs* liver after intratracheal instillation of 25 μg/kg of TAP in neonatal rats.

B



C

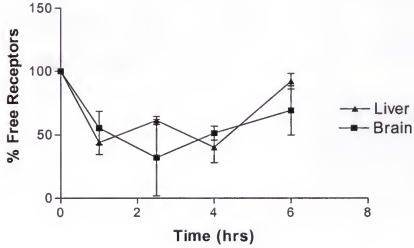
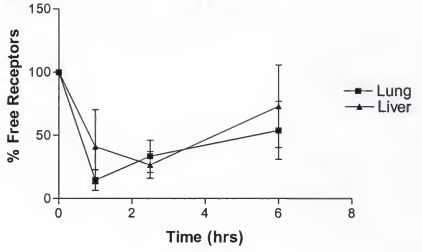


Fig 3-2: Continued

A



B

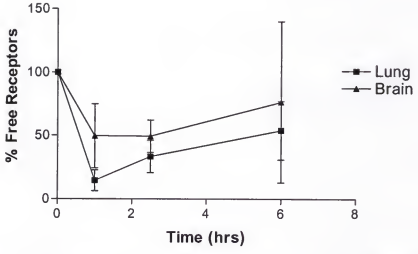


Fig 3-3: Percent free receptors *vs* time profiles in (A) lung *vs* liver (B) lung *vs* brain and (C) brain *vs* liver after intratracheal instillation of 50 μg/kg of TAP in neonatal rats.

C

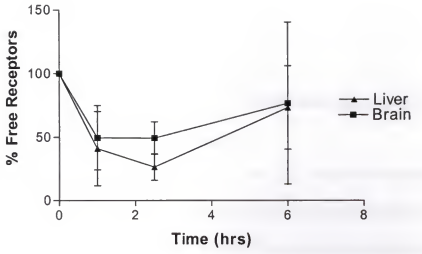


Fig 3-3: Continued

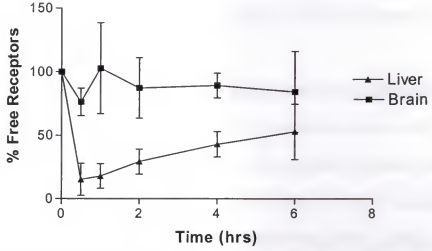


Fig 3-4: Percent free receptors *vs* time profiles in the brain and liver of adult rats after intratracheal instillation of 100 µg/kg TA (data taken from reference(41)).

Table 3-1: Average AUC estimates in the lung, liver and brain after intratracheal administration of 2.5, 25 and 50 μg/kg of TAP to neonatal rats and 100 μg/kg to adult rats.

		Average AUC (%)		
Dose (μg/kg)	N	Lung	Liver	Brain
2.5	4	18.3 ± 4.5	17.4 ± 13.5	-14.7 ± 11.9
25	2	36.3 ± 12.6	45.3 ± 7.8	45.7 ± 9.7
50	4	59.9 ± 9.4	50.8 ± 13.0	47. ± 9.6
100*	3	-	63.1 ± 8	11.3 ± 6.7

* 100 μg/kg of TA was administered intratracheally to adult rats, data from reference (41)

Discussion

A previously developed model by Hochhaus et al. (55) was used to simultaneously assess the fraction of receptors occupied in the local (lung) and systemic (liver, brain) organs after different doses of TAP in neonatal rats. The assay is a radioligand binding assay which is used to monitor the decrease in % free receptors (increase in receptor occupancy) as a function of time. Using this functional assay, we could determine the receptor occupancy of TAP in different organs (lung, liver and brain) as a function of time. TAP is a prodrug of triamcinolone acetonide (TA) and is efficiently metabolized to TA (85).

We did not see appreciable pulmonary targeting after administering TAP solution at the different doses ($p > 0.05$). This can be attributed to the rapid absorption of the TAP solution from the lungs into the systemic circulation resulting in similar pulmonary and hepatic receptor occupancies. In fact, Hochhaus et al. (52), through a series of computer simulations, have shown that the rapid removal of the dissolved drug from the lungs and its absorption into the systemic circulation results in similar pulmonary and systemic drug levels leading to negligible pulmonary targeting. The results obtained from the computer simulations have been experimentally corroborated by Talton et al. (41) and Suarez et al.

(63) for a wide variety of inhaled corticosteroids. In addition, we did not observe a clear dose response relationship between different doses of TAP administered and the AUC's obtained in the lung and liver. This can be due to saturation of the corticosteroid receptors in the lung and liver at the different doses used. The log-linear nature of the dose response relationship can explain the non-linearity observed between the doses of TAP administered and the response (average AUC's) obtained in the different organs of neonatal rats.

We used 10-11 days old rats as the neonatal rat model in our study. The pattern of brain development is highly species specific. In mammals such as guinea pig and primates, the majority of neurodevelopmental processes are completed *in utero* (86, 87). However, in animals which give birth to immature young ones such as rats and mice, the major portion of neurodevelopment takes place after birth (88). Consequently, the neonatal rat model used in our study represents a valid model to assess the pharmacologically relevant concentrations of the corticosteroid in the brain that might be linked to the neurotoxic adverse effects of corticosteroids in preterm infants.

An interesting and important observation from the dose response studies was that TAP showed receptor occupancy in the brain of neonatal rats. As previously noted, similar receptor binding studies performed in adult rats by Saurez et al. (89), Talton et al. (41) and Wang et al (42) after have shown the absence of brain receptor occupancy in adult rats irrespective of the corticosteroid used (budesonide, fluticasone propionate, triamcinolone acetonide) and the route of administration (intravenous, intratracheal).

The lower brain receptor occupancy observed in adult rats after intratracheal instillation of TA powder can be due to the higher hepatic clearance of the corticosteroid

from the systemic circulation. The efficient removal of the drug from the systemic circulation in adult rats (due to well developed hepatic system) can result in less drug available for entering the brain. On the other hand, the incomplete development of hepatic metabolic pathways in neonatal rats can lead to higher systemic levels and consequently higher availability of the drug to enter the brain. However, the *ex vivo* receptor binding assay used simultaneously tracks the receptor occupancy in the local (brain) and systemic (liver) organs. Our results show pronounced liver receptor occupancy in adult rats that seem to suggest that higher clearance in adult rats cannot explain the differences in brain receptor occupancy between neonatal and adult rats.

A potential reason for differences in brain receptor occupancies between neonatal rats and adult rats can probably be due to the lack of a functional blood brain barrier in neonatal rats. In addition, the higher brain receptor occupancy in neonatal rats suggests the absence of a fully matured blood brain barrier. This absence of a fully matured blood brain barrier can be one of the likely explanations for the neurotoxic adverse effects observed in preterm infants after systemic corticosteroid administration.

Another reason for observing higher brain receptor occupancy in neonatal rats can be the absence of fully functional p-gp transporters (due to immaturity of the blood brain barrier). As described in chapter 2 , the results from studies with wild type and mdr1a (-/-) mice have explicitly shown that after intravenous administration of TAP (100 μg/kg) to knockout mice and wild type mice, there is a significantly higher brain receptor occupancy in knockout mice as compared to wild type mice (90). Matsuoka et al. (91) have studied the expression of p-gp transporters in the brain of rats as a function of gestational age. It was shown that p-gp was undetectable until postnatal day 7, after

which the p-gp expression showed a steady increase to reach a plateau at day 20 with about 25 % development at day 10. Since the neonatal rats used in our study were 10-11 days old, there is a strong possibility that the enhanced permeability of TAP in the brain of neonatal rats (resulting in significantly higher brain receptor occupancy) was a consequence of incomplete development of the p-gp transporters (due to immaturity of the blood brain barrier). The presence, albeit insignifant, of p-gp transporters in 10 day old rats can probably explain the absence of brain receptor occupancy (due to active extrusion of TAP by the p-gp transporters) at the lowest dose used in our study (2.5 μg/kg). Further, the negative brain receptor occupancy observed at the lowest dose was most likely due to the inherent variability of the assay used. Although there was significant brain receptor occupancy at higher doses (25 and 50 μg/kg) as compared to the lowest dose, the absence of a linear relationship between the higher doses of TAP administered and the brain receptor occupancy can be attributed to saturation of p-gp transporters resulting in reduced efflux of TAP. This further indicates the critical role played by p-gp in modulating the permeability of corticosteroids across the blood brain barrier.

The presence of fully functional p-gp transporters (due to fully developed blood brain barrier) in adult rats can possibly explain the minimal brain receptor occupancy observed after intratracheal insitllation of TA (fig 3-4). This active efflux by p-gp results in very low pharmacologically relevant brain concentrations. In addition, similar results have been reported by deKloet et al. (40) who have shown that dexamethasone poorly penetrates the brain of adult rats. Although our results, together with the results of Matsuoka et al. implicate the poor development of p-gp transporters on the blood brain

barrier as one of the major factors for increased permeability of TAP in neonatal rats, more conclusive biochemical studies need to be performed to study the expression of p-gp transporters in preterm infants. This would lead to a significant understanding of the role played by p-gp transporters in modulating the permeability of corticosteroids in humans. The information on the expression of p-gp transporters as a function of gestational age can be utilized for making detailed dosing recommendations for antenatal/postnatal corticosteroid therapy in preterm infants suffering from CLD.

Conclusion

- A previously developed *ex vivo* receptor binding method was successfully adapted in neonatal (10-11 days old) rats to simultaneously monitor the glucocorticoid receptor occupancy in the local (lung) and systemic (liver, brain) organs after intratracheal instillation of different doses of TAP.
- We did not observe a clear dose response relationship between different doses of TAP used and the average AUC estimates obtained in the lung, liver and brain. This can probably be due to saturation of glucocorticoid receptors at the various doses used and the log linear relationship between dose and response.
- The results show significantly higher brain receptor occupancy at higher doses in neonatal rats than adult rats thereby suggesting the lack of a functional blood brain barrier in preterm infants. This is in close agreement with the observance of adverse effects in preterm infants after systemic corticosteroid administration.
- We did not observe brain receptor occupancy in adult rats after intratracheal instillation of TA. This can be attributed to the active efflux of TA by p-gp transporters.

- The results from our study underscore the important and urgent need to develop targeted delivery systems to the lungs for administering inhaled corticosteroids to preterm infants. This will greatly assist in increasing the local effect of steroid in the lungs and reduce the systemic spill over thereby increasing the benefit to risk ratio.

CHAPTER 4
PULMONARY TARGETING OF SUSTAINED RELEASE FORMULATION OF BUDESONIDE IN NEONATAL RATS

Introduction

The delivery of corticosteroids through the inhalation route for the treatment/prevention of chronic lung disease has gained attention in recent years. However, as previously noted, a number of studies have also shown the limited effectiveness of inhaled glucocorticoid therapy in premature infants suffering from CLD (47, 48).

The limited success of inhaled corticosteroid therapy in preterm infants can be, in part, explained on the basis of rapid absorption of lipophilic corticosteroids across the high absorptive surface provided by the pulmonary epithelium (83). This rapid absorption from the lungs into the systemic circulation results in very low pharmacologically active pulmonary drug concentrations and low pulmonary residence time (the time for which the drug stays in the lung before being absorbed into the systemic circulation). This leads to adverse systemic effects and a lower benefit to risk ratio. Hence, alternative strategies for drug delivery are urgently required which will increase the pulmonary residence time of the drug thereby increasing the desired local effects with concomitant reduction in systemic exposure.

Recently, the use of pulse laser deposition (PLD) technique to coat drug particles with nano thin films (thereby significantly reducing the polymer load) of biodegradable polymers such as poly (l-lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) has

gained widespread attention (92, 93). The polymer coated formulation thus obtained is expected to sustain the release of the drug powder thereby leading to higher pulmonary residence time and improved pulmonary targeting. Talton et al. (93) have performed *in vitro* (using dissolution tests) and *in vivo* (using *ex vivo* receptor binding assay) characterization of PLGA coated budesonide and PLA coated triamcinolone acetonide dry powders. They showed that the half-life of release ($t_{50\,\%}$) of polymer coated budesonide was significantly higher as compared to uncoated budesonide (60 ± 1.6 min *vs* 1.2 min). Using a previously developed *ex vivo* receptor binding assay (55), it was shown that the alteration in dissolution behavior of the coated budesonide translated into significant improvement in pulmonary targeting.

Hypothesis

We hypothesize that the pulmonary instillation of PLA coated budesonide in neonatal (10-11 days old) rats will also result in sustained lung receptor occupancy and a higher degree of pulmonary targeting as compared to uncoated budesonide. To test our hypothesis, e*x vivo* receptor binding assays were performed in neonatal (10-11 days old) to track the % free receptors in the lung, liver and brain after intratracheal administration of uncoated/polymer coated budesonide. The average receptor occupancies ($AUC_{0\text{-}6\,h}$ / 6) in the lung, liver and brain and the pulmonary targeting (defined as AUC_{lung}/AUC_{liver}) were obtained from the % free receptors *vs* time profiles.

Materials and Methods

Micronized BUD was obtained from Astra Zeneca Pharmaceuticals (Wilmington, DE). Extra fine lactose monohydrate was donated from EM industries (Hawthrone, NY). Phosphate buffered saline (PBS) (pH 7.4) was obtained from Cellegro® (Mediatech, Herndon, VA). ({6,7-^{3}H} dexamethasone, 35-40 Ci/mmol) was obtained from New

England Nuclear (Wilmington, DE). All other chemicals were obtained from Sigma (St. Louis, MO) or equivalent sources.

Preparation of Uncoated/PLA coated Budesonide Suspensions and Radiolabelled Solutions

0.4 % of the uncoated/ PLA coated budesonide powders were prepared in extrafine lactose. Approximately 6.25 mg of the powders (equivalent to 25 μg of the free drug) were weighed in a 1.5 ml tubes. 300 μl of the PBS was added prior the administration. This suspension was intratracheally administered (50 μg/kg) to the neonatal rats.

25 nM ^{3}H labeled dexamethasone was prepared in incubation buffer (mixture of 10 mM Tris/HCl and 10 mM sodium molybdate in cold water) and used as tracer solution. A mixture of 25 nM ^{3}H labeled dexamethasone and 25 μM unlabelled dexamethasone, prepared in incubation buffer, was used to estimate the non-specific binding.

Coating Procedure

The PLA polymer target was prepared in a Carver Press®(Wabash, IN). One gram of polymer was weighed, transferred into a 1 inch × 0.25 inch circular mold and pressed with 2500 psi at 100 °C for 10 min. A pulsed excimer laser using Krypton Fluoride source (λ=248 nm) was used to ablate the polymer in a vacuum chamber. A 5 Hz laser frequency was used to perform the ablation. The polymer was ablated onto 100 mg of fluidized micronized BUD within the same chamber for 1 h. The coating procedure was performed by personnel at the Engineering Research Center (ERC), University of Florida.

Animal Procedures

All animal procedures were approved by the institutional animal care and use committee, (IACUC), University of Florida, an Association for the Assessment and

Accreditation of Laboratory Animal Care (AAALAC) approved facility. Neonatal rats (20 ± 5 g) were obtained from Harlan (Indianapolis, Indiana). The neonatal rats were anesthetized with an anesthetic mixture (1.5 ml of 10 % v/v ketamine, 1.5 ml of 2 % v/v xylazine and 0.5 ml of 1 % v/v acepromazine) at the dose of 1 ml/kg. The skin on the neck was shaved and the area cleaned with betadine solution. A 1-cm incision was made in the skin with a sterile scalpel blade to expose the underlying musculature. The muscles were gently teased apart with a sterile curved hemostat to expose the trachea. An incision was made between a pair of tracheal rings and uncoated/coated budesonide (50 µg/kg) suspension was intratracheally administered. The placebo rats were administered saline. Following surgery, the rats were placed on a fresh drape overlying a heating pad and were kept warm with the aid of a heating pad. The rats were decapitated at 1, 2.5, and 6 h and the lung, liver and brain were removed. The weighed tissue was added to 10 times (for liver) and 4 times (for lung and brain) organ weight of ice-cold incubation buffer (10 mM Tris/HCl, 10 mM sodium molybdate, 2 mM 1,4-dithioerythritol). The homogenate was incubated with 5 % charcoal (in distilled water) for 10 minutes to remove endogenous corticosteroids. Aliquots of the supernatant (150 µL) were added to pre chilled microcentrifuge tubes containing 50 µL of either 25 nM ^{3}H labeled dexamethasone for determining the total binding or a mixture of 25 nM ^{3}H labeled dexamethasone and 25 µM of unlabelled dexamethasone for determining the non-specific binding. The microcentrifuge tubes were vortexed and incubated at 4 ° C for 18 h.

After the incubation, 200 µL of activated charcoal (5 % in water) was added to the microcentrifuge tubes to remove excess radioactivity. The microcentrifuge tubes were

vortexed, centrifuged for 5 minutes and 300 μL of the supernatant was removed and added to the scintillation vial. 5 mL of the scintillation cocktail (Cytoscint™, ICN Biomed, Costa Mesa, CA) was added and the scintillation vials were read in a scintillation counter (Beckman, LS 5000TD, Palo Alto, CA) to obtain the radioactive counts (measured in disintegration per minute (dpm's)) in various tissues.

For a given tissue (lung, liver or brain), the radioactivity counts (measured in dpm's) corresponded to the total binding (specific + non specific) of the tracer. The dpm's corresponding to the non-specific binding (obtained from incubating the cytosol with a mixture of 25 nM ^{3}H labeled dexamethasone and 25 μM unlabelled dexamethasone, removing excess radioactivity and determining the radioactive counts in the supernatant) was subtracted from the total binding to obtain estimates of the specific binding. The specific binding obtained in the rats administered saline (placebo) corresponded to 100 % free receptors. The % free receptors present in the brain or liver was calculated as

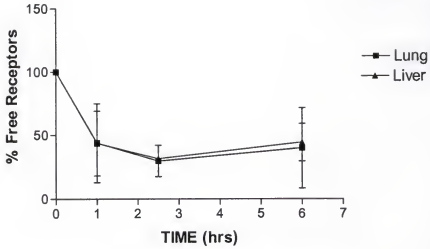
$$\% \text{ free receptors in a tissue} = \frac{\textit{specific binding in a tissue of rat administered TAP}}{\textit{specific binding in a tissue of rat administered saline}} * 100$$

For each tissue, the cumulative $AUC_{0\text{-}6h}$ calculated from the % free receptors *vs* time profile was subtracted from 600 (each hour of the experiment corresponds to 100 % free receptors) to obtain the cumulative $AUC_{0\text{-}6h}$ for % bound receptors. The average receptor occupancies (AUC) in the brain and liver for wild type and mdr1a mice were obtained by dividing the cumulative receptor occupancy ($AUC_{0\text{-}6h}$) by 6h (the duration of experiment). The differences in pulmonary and hepatic receptor occupancies (AUC_{lung}-AUC_{liver}) after different doses were compared using student t test.

Results

Fig 4-1 (A-B) shows the % free receptors as a function of time in the lung and liver of neonatal rats after administration of uncoated and polymer coated budesonide respectively. Fig 4-2 (A-B) shows the % free receptors as a function of time in the lung and brain of neonatal rats after administration of uncoated and polymer coated budesonide respectively. Table 4-1 shows the pulmonary targeting and the average receptor occupancy estimates obtained in the lung, liver and brain of neonatal rats after intratracheal administration of uncoated/coated budesonide. The average receptor occupancy in the lung, liver and brain after intratracheal administration of micronized uncoated budesonide were 58.4 ± 12.9 %, 56.4 ± 6.8 % and 38.3 ± 6.7 %. However, after administration of PLA coated budesonide, the average AUC estimates in the lung, liver brain were 75.8 ± 3.7 %, 46.6 ± 14.5 % and 29 ± 7 %. The average receptor occupancies in the lung and liver after administration of uncoated budesonide were similar ($p>0.5$). However, the average lung and liver receptor occupancies after administration of PLA coated budesonide were significantly different ($p < 0.05$). The pulmonary targeting (AUC_{lung}/AUC_{liver}) after intratracheal administration of uncoated budesonide was 1.03 ± 0.13 and 1.72 ± 0.46 respectively.

A



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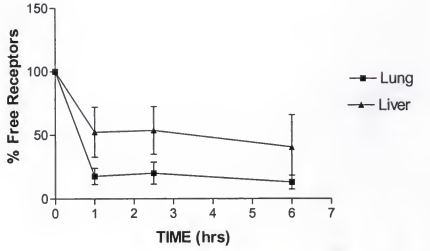
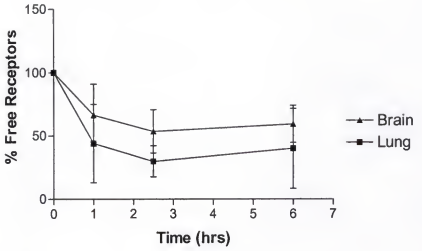


Fig 4-1: Percent free receptors *vs* time in the lung and liver of neonatal rats after intratracheal instillation of (A) micronized uncoated budesonide and (B) PLA coated budesonide (50 μg/kg).

A



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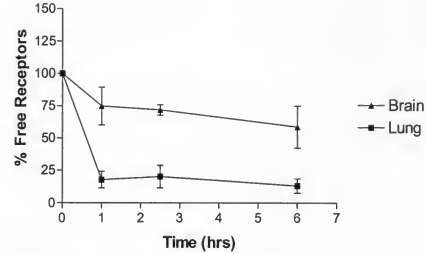

Fig 4-2: Percent free receptors *vs* time in the lung and brain of neonatal rats after intratracheal instillation of (A) micronized uncoated budesonide and (B) PLA coated budesonide (50 μg/kg).

Table 4-1: Average AUC's (n=3) in the lung, liver and brain and pulmonary targeting (PT) in neonatal rats after intratracheal administration (50 μg/kg) of uncoated budesonide and PLA coated budesonide.

Formulation	Dose (μg /kg)	Average AUC (%)			PT (AUC_{lung}/ AUC_{liver})
		Lung	Liver	Brain	
Uncoated Budesonide	50	58.4 ± 12.9	56.4 ± 6.8	38.3 ± 6.7	1.03 ± 0.13
PLA Coated Budesonide	50	75.8 ± 3.7	46.6 ± 14.5	29 ± 7	1.72 ± 0.46

Discussion

The enthusiasm of using inhaled corticosteroid therapy in preterm infants for the treatment/prevention of chronic lung disease has simmered due to the observance of extrapulmonary adverse effects. Hence, treatment strategies need to be developed which can improve the clinical effectiveness (topical efficacy:systemic activity) of inhaled corticosteroids.

The last few years have witnessed the development of inhaled corticosteroids such as budesonide and fluticasone propionate based on optimized pharmacokinetic properties. Ideally, an inhaled corticosteroid should produce therapeutic effect at the pulmonary site, should have minimum oral bioavailability and should be rapidly cleared once it is absorbed into the systemic circulation. In addition to all these factors, another parameter that is responsible for achieving improvement in pulmonary selectivity is the pulmonary residence time.

The pulmonary residence time is governed by a combination of factors such as the release rate of the drug (from the powder/delivery system), rate of absorption into systemic circulation and the pulmonary clearance of the drug by the mucociliary transporters. Computer simulations have shown that rapid release of the drug (incase of solutions) results in fast absorption leading to similar pulmonary and systemic drug levels

and loss of pulmonary targeting (expressed as the difference between pulmonary and systemic receptor occupancies) (52). As the dissolution rate (release rate) is decreased, the pulmonary targeting increases and reaches a maximum at an "optimal" dissolution rate. Further reduction is dissolution rate leads to pulmonary clearance of a major portion of the drug *via* the mucociliary transporter before the drug can show its therapeutic effect. Hence, a sustained release system optimized for the release rate can potentially lead to pronounced pulmonary selectivity.

As previously noted, a variety of approaches such as slowly dissolving drug particles, intracellular formation of esters (incase of budesonide) and slow release systems such as liposomes and microspheres can be employed to increase the pulmonary residence time. The *in vivo* utility of slow release systems to increase the pulmonary residence time has also been emphasized using animal models. Gonzales rothi et al. (62) have shown that pulmonary instillation of liposomal encapsulated triamcinolone acetonide phosophate (TAP) resulted in increased pulmonary residence time thereby leading to improved pulmonary targeting . Similarly, Brattsand et al. (64) have reported increase in pulmonary selectivity with budesonide palmitate liposomes. However, limitations such as leakage of encapsulated material and low encapsulation efficiencies have limited the use of liposomes as model systems for demonstrating pulmonary targeting. In order to overcome these formulation related limitations, we used the PLD method for preparing polymer coated sustained release formulation of budesonide.

As budesonide has been shown to rapidly dissolve in the lungs of rats (94) and humans (95), the pulmonary administration of budesonide by a sustained release delivery

system is expected to increase the pulmonary residence time and improve pulmonary targeting.

We compared the receptor occupancy in the lung, liver and brain of neonatal (10-11 days old) rats after intratracehal administration of PLA coated/uncoated formulations of budesonide using an *ex vivo* receptor binding assay. The assay is a surrogate marker for pharmacologically active free drug concentrations in various tissues, hence determining the receptor occupancy in various tissues will help in the indirect assessment of local (lungs) and systemic (liver and brain) corticosteroid exposure.

We did not observe significant pulmonary targeting after intratracheal administration of uncoated budesonide that can be attributed to the rapid absorption of budesonide from the lung into the systemic circulation leading to similar local and systemic exposure. However, we observed significant brain receptor occupancy after intratracheal administration of uncoated budesonide. These results are in good agreement with the our previous results (chapter 3) where we observed significant brain receptor occupancy in neonatal rats after intratracheal instillation of triamcinolone acetonide phosphate, an inhaled corticosteroid (96). Further, the incomplete development of the p-gp transporters (due to an immature blood brain barrier) can, in part, explain the observance of significant brain receptor occupancy. Matsuoka et al. (91) have shown that that the development of p-gp transporters in rats starts at day 7 and steadily increases to reach a plateau at day 20 (with about 25 % developed at day 10). As the rats used in our study were 10-11 days old, the significant brain receptor occupancy can probably be due to poor development of the p-gp transporters (due to an immature blood brain barrier).

We observed significant pulmonary targeting after intratracheal administration of PLA coated budesonide. This can probably be explained on the basis of an increase in the pulmonary residence time of the polymer-coated formulation leading to sustained receptor occupancy. Although the brain receptor occupancy after intratracheal administration of polymer-coated budesonide was lower as compared to uncoated budesonide, the results were not significantly different to make any conclusions regarding the differences in brain exposure. However, sustained receptor occupancy (resulting in higher pulmonary targeting) observed after intratracheal administration of polymer-coated budesonide can lead to reduction in the dose of corticosteroid administered. This will potentially result in the reduction of systemic exposure.

Conclusion

- The results from our study show that the pulmonary targeting in neonatal rats was significant improved by using polymer-coated slow release formulation of budesonide.
- The significant improvement in pulmonary targeting potentially allows for a reduction in dose administered thereby leading to reduction in systemic adverse effects.
- The efficacy (by increase in pulmonary residence time) and safety (by administering lower doses of the glucocorticoids resulting in less "spill over" into systemic circulation) of inhaled glucocorticoids can be improved by use of optimally designed slow release formulations.

CHAPTER 5
CONCLUSIONS

Systemic corticosteroids are widely used for the treatment/prevention of chronic lung disease (CLD). Although systemic corticosteroids have shown beneficial effects, the concomitant adverse effects have simmered the enthusiasm for using them. The last few years have witnessed the use of inhaled steroids for the prevention/treatment of CLD. However, inhaled corticosteroid therapy has met with limited success, partly due to high lipophilicity of commercially available corticosteroids resulting in rapid absorption of the corticosteroid from the lungs into systemic circulation. This rapid absorption leads to significant reduction in the pulmonary residence time and consequently, a loss of pulmonary targeting.

The overall objective of this thesis was to study the biopharmaceutical factors that modulate the disposition of inhaled corticosteroids in preterm infants. In addition, the usefulness of microencapsulated corticosteroid formulations for increasing the pulmonary targeting was evaluated.

In the first set of experiments, we investigated the role played by p-gp transporters in modulating the brain permeability of inhaled corticosteroids in mice. The brain and liver receptor occupancies were determined in wild type and mdr1a mice after intravenous administration of TAP. The results showed significantly higher brain receptor occupancy in mdr1a mice that underscores the critical role played by p-gp transporters in modulating the brain permeability of inhaled steroids. Previous studies

performed in our laboratory have shown minimal brain receptor occupancy in adult rats. Hence, our results, taken in conjugation with previous studies, suggest that p-gp transporters modulate the brain permeability of all clinically relevant inhaled corticosteroids.

The next set of experiments involved the simultaneous monitoring of corticosteroid receptor occupancy in the local (lung) and systemic (liver, brain) organs of neonatal rats after intratracheal instillation of different doses of triamcinolone acetonide phosphate (TAP). This was done primarily to determine if pulmonary instillation of TAP demonstrates pulmonary targeting in neonatal rats. As expected, we observed similar pulmonary and hepatic receptor occupancies (no pulmonary targeting) that can be attributed to the rapid absorption of TAP into systemic circulation. However, an interesting and important observation from these experiments was that TAP showed brain receptor occupancy in the neonatal rats. This was in sharp contrast to minimal brain receptor occupancy observed in adult rats observed in previous similar studies performed in our laboratory. This higher brain receptor occupancy in neonatal rats (and absent in adult rats) can probably be explained on the basis of an immature blood brain barrier in neonatal rats. In addition, the poor development of p-gp transporters (due to an immature blood brain barrier) in neonatal rats can also, in part, explain the increased permeability of the corticosteroid.

In our last set of experiments, we evaluated the use of a novel sustained release drug-delivery system for improving the pulmonary targeting of budesonide, a widely used inhaled corticosteroid. Poly (l-lactic acid) coated budesonide and uncoated budesonide was intratracheally administered to neonatal rats and the degree of local

(lungs) and systemic (liver, brain) corticosteroid receptor occupancies were determined using *ex vivo* receptor binding assay. The results showed significantly higher pulmonary targeting after intratracheal administration of polymer-coated budesonide as compared to uncoated budesonide. The significant differences in pulmonary targeting can potentially help to reduce the amount of dose. This can lead to reduction in systemic adverse effects observed after corticosteroid administration thereby improving the benefit to risk ratio.

Overall, the results from our study underscore the important and urgent need to develop targeted delivery systems to the lungs for administering inhaled corticosteroids to preterm infants. This will greatly assist in increasing the local effect of steroid in the lungs and reduce the systemic "spill over" thereby increasing the benefit to risk ratio.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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